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Variation of the Savi's warbler (*Locustella luscinioides*) leucocyte profiles and body condition in relation to age, sex and moult

Izabela Kulaszewicz*, Katarzyna Wojczulanis-Jakubas & Dariusz Jakubas

*University of Gdańsk, Department of Vertebrate Ecology and Zoology, ul. Wita Stwosza 59, PL-80-308 Gdańsk, Poland (*corresponding author's e-mail: izabela.kulaszewicz@ug.gda.pl)*

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Birds of different sex and age are known to differ in morphology and physiology. We investigated leucocyte profiles and body condition of the Savi's warbler (*Locustella luscinioides*) in relation to sex, age and moulting status. We caught birds during the post-breeding period at the stopover site in northern Poland. Of the leucocyte profiles, we examined the heterophil/lymphocyte ratio (H/L), and the number of leucocytes, lymphocytes and heterophils per 10 000 red blood cells (RBC). We found a significant effect of the pentad × age interaction on the H/L ratio. In adults, body condition (BC, size-adjusted body mass) was similar in both sexes. Among adults, females had higher numbers of leucocytes and heterophils per 10 000 RBC and a lower relative number of lymphocytes compared to males. It is possible that subtle sex differences in parental care at the end of the breeding period account for these differences. They may be too subtle to affect BC, but are visible at some leucocyte variables. Moreover, BC of adults decreased with date, indicating the presence of migratory birds using our study area as a stopover site. Moulting adults had higher numbers of all leucocytes and lower H/L values than unmoulted individuals, suggesting a lack of trade-off between the immune system and moulting.

Introduction

The main three energy-demanding events in the annual cycle of birds, i.e. breeding, moult, and migration, are strongly reflected in physiology and condition (Drent & Daan 1980, Norris & Evans 2000, Sheridan *et al*. 2004). Generally, these important life-history events tend to occur at different times of the year with minimal overlap. The timing of these events as well as their sequence in the annual cycle are assumed to be shaped by selective forces (likely environmental conditions), ensuring that each event occurs at an optimal time of the year (Gosler 1996, Visser *et al*. 2004, Barta *et al*. 2008, Milenkaya *et al*. 2013) and minimizes competition between these processes for limited resources, which could, in turn, compromise the outcome of each (Barta *et al*. 2008, Wingfield 2008). Reproduction is estimated to increase daily basal metabolic rate by approximately 25%–50% (Monaghan & Nager 1997, De Heij *et al*. 2008), migration by

20%–25% (Lindström *et al.* 1999), and moult by 10%–45% (Buttemer *et al.* 2003, Hoye & Buttemer 2011). Of these, moulting is the most variable in time and may overlap with other important life-history events in some species (Echeverry-Galvis & Hau 2013).

Energy allocation into a particular life history event may differ among various sex and age groups. For example, blood variables and body condition (hereafter considered as body mass adjusted for body size) may differ between the sexes due to various costs of gamete production, parental roles, contributions made into parental duties and territory defence, or different rates and strategies of moulting (Ots *et al.* 1998, Sanz *et al.* 2000, Pap *et al.* 2010). Also, the age of birds may affect values of blood variables and body condition significantly due to differences in experience and social status (Woodrey & Moore 1997). Different moulting patterns of adults and immatures may also affect values of blood variables (Quillfeldt *et al.* 2008, Pap *et al.* 2010) as a result of age differences.

In the present study, we investigated leucocyte profiles, body condition and fat reserves in the Savi's warbler (*Locustela luscinioides*) in relation to age, sex, date (progress of the post-breeding period and migration) and moult status. This species is a territorial, migratory passerine. It breeds in marshlands with reeds and sedges in Europe and Asia, and winters in sub-Saharan Africa. Females lay four to six eggs. Incubation lasts about 12 days and involves both parents. Both sexes feed chicks with similar frequency (Pikulski 1986, Cramp 1998). Post-breeding moult of adults may be completed before autumn migration or suspended (within primaries and secondaries) (Svensson 1992). The extent and timing of post-breeding moult is variable (Kulaszewicz & Jakubas 2015). Some birds start moulting at stopover sites in northern Africa before moving further south, or even at winter quarters (Pikulski 1986, Jenni & Winkler 1994, Cramp 1998). Immatures undergo a partial post-juvenile moult (replacing all covert and body feathers) prior to the autumn migration (Svensson 1992).

Our main objectives were to investigate: (1) how age, sex and date (progress of the post-breeding period and migration) affect leucocyte profiles, body condition and fat reserves of Savi's warblers during the post-breeding period; (2) how the post-breeding moult affects leucocyte profiles and body condition of adults, and whether there is a trade-off between immune function and moulting; and (3) how date affects energy reserves (body condition and fat scores).

We hypothesized that during the post-breeding period, adults should have higher stress levels as compared with immatures due to physiological costs incurred during the breeding season. However, we expected that adults, due to their experience and effectiveness in obtaining food, should have better body condition and higher fat reserves than immatures. We expected that adult males and females should not differ significantly in body condition, fat reserves and leucocyte profiles, considering similar parental roles of both sexes. In light of high energy demands of moulting and possible trade-offs with reproduction and/or migration, moulting adults are expected to have lower values of leucocyte profiles, worse body condition and lower fat reserves. Finally, we expected to find an increase in body condition and fat reserves with capturing date in all individuals, because of progress in time towards the autumn migration and related increased feeding and accumulation of reserves.

Material and methods

Field work

We captured birds in the southern part of the "Lake Druzno" reserve (54°05´N, 19°27´E) in northern Poland. Lake Druzno is a large, shallow lake overgrown by reedbeds and swampy vegetation (Nitecki *et al.* 2013). It is an important breeding and stopover site for passerines associated with wetlands (Jakubas & Wojczulanis-Jakubas 2010). We captured birds in 20 mist-nets (Ecotone, Poland) situated in reedbeds during the post-breeding season, corresponding to post-breeding dispersal and autumn migration in 2010 (25 July–21 August) and 2011 (25 July–28 August). We ringed all birds and aged them as adults (≥ 2 years) or immatures (1 year) according to plumage and iris colour (Svensson 1992).

We measured head-bill length (with a calliper, 0.1 mm accuracy) and weighed birds with an electronic balance (Ohaus, with 0.1 g accuracy). Re-captured individuals were not included in the analyses. To estimate fat reserves, we used the 0–8 fat score scale (after Busse 2000) and calculated the median values and interquartile ranges (Q_1–Q_3). In the present study, we observed only scores 0–4:

0 belly without visible fat, air-sack is visible within the furculum;
1 belly without visible fat or with reddish traces only, the whole interior of the furculum covered with fat;
2 belly with infused bands of fat (visible intestine), *furculum* not inspected;
3 belly with fused fat covering the intestine but not the liver, *furculum* not inspected;
4 belly completely covered with fat, fat in the *furculum* flat or concave.

To estimate moult extent in adults, we used a moult score, assigning a score from 0 (old feather) to 5 (completely grown feather) to all primary feathers (Ginn & Melville 1983). We numbered primaries from the inner to the outer part of the wing, assigning each feather a sequential number from 1 to 9 (P1–P9). All measurements were done by the same person (IK).

We took a small blood sample (10–20 μl) from the brachial vein (Owen 2011) for preparing a blood smear for leucocyte profiles and molecular sexing. We sampled each individual only once. We stained air dried blood smears using the May-Grünewald-Giemsa method with a Wescor "Aerospray Haematology" stainer. We determined numbers of particular types of leucocytes by examining one-layer-cells, non-overlapping microscope fields of each smear at 1000× magnification under oil immersion. All types of leucocytes (heterophils, lymphocytes, basophiles, eosinophils and monocytes) were counted until reaching a total of 100 cells. Moreover, we counted the number of heterophils, lymphocytes and all types of leucocytes per 10 000 red blood cells (RBC) (Lobato *et al*. 2005). All blood smears were examined by a single observer, oblivious of the bird's age, sex and moulting status (IK).

Since neither plumage nor morphometric variables discriminate sex in all Savi's warblers accurately (Kulaszewicz *et al*. 2013), we identified the sex of captured individuals molecularly (Griffiths *et al*. 1998). We extracted DNA following the evaporation of alcohol and using the Blood Mini kit (A&A Biotechnology, Gdynia, Poland). We performed sexing by amplification of a 390-bp fragment of the CHD gene on the W chromosome (in females only), and a 370-bp fragment on the Z chromosome (in both sexes), using the primer pair P2 and P8, universal for birds (Griffiths *et al*. 1998). We performed PCR according to the protocol described by Griffiths *et al*. (1998) with 50 °C for annealing the primers. Difference in size of the PCR products was clearly visible in UV light on a 2% agarose gel stained with ethidium bromide.

In total, we captured, measured and blood-sampled 66 adult (30 males and 36 females) and 151 immature (95 males and 56 females) Savi's warblers. Of that total, 30 adults and 69 immatures were captured in 2010 and 36 adults and 82 immatures in 2011.

Variables studied

Leucocyte profiles are widely used to investigate changes in nutritional state (e.g. Merilä & Svensson 1995, Alonso-Alvarez & Ferrer 2001), reproductive efforts (e.g. Merino & Barbosa 1997) and health status (Fargallo *et al*. 2001, Wojczulanis-Jakubas *et al*. 2012, Wojczulanis-Jakubas *et al*. 2014) of birds. The ratio of the two most common leucocytes, heterophils and lymphocytes (hereafter the H/L ratio) has been described as a measure of stress in birds (Ots & Hõrak 1996, Vleck *et al*. 2000, Davis *et al*. 2008). Leucocyte profiles are influenced by physiological changes in the annual life cycle of birds. Breeders usually have a higher H/L ratio because brood rearing may impose a greater work load and exert greater stress on birds (Hõrak *et al*. 1998). Elevated H/L ratios in birds are also associated with increased immunosuppression (Ots & Hõrak 1996). Moulting birds usually have a lower H/L ratio because of the redirection of heterophils to tissues within the forming of new feathers; to prevent bacterial infection to which the organism is exposed during this process (Norris & Evans 2000, Driver 1981, Møller & Saino 2004). Immune function is suppressed in response to elevated energetic demands of migration (Owen & Moore 2006). The number of leucocytes per 10 000 red blood cells (RBC) may provide information on the health and immunocompetence of an individual bird (Campbell & Dein 1984). Changes in numbers of leukocytes also reflect ongoing disease processes of bacterial, parasitic, or viral origin (Hoffman-Goetz & Pedersen 1994).

Body condition (body mass adjusted to body size) and fat load are the simplest and most wildly used indices of nutritional state. Heavier individuals, with greater fat reserves, are considered to be in better nutritional state. It has been suggested that heavier individuals also have a stronger immune system (Alonso-Alvarez & Tella 2001), higher reproductive success (Garnett 1981) and higher survival rate during migration (Lindström & Piersma 1993). Fat is the main energy source used by birds to fuel them during migration (Hedenström *et al.* 2009). The accumulation of fat loads is probably a result of trade-offs between several costs and benefits (e.g. migration speed, predation risk). The investigation of fat load at stopover sites along the migration route may thus be useful for characterizing migration strategies (Lindström & Piersma 1993).

Data analyses

As a body condition index, we considered the scaled mass index, i.e. a bird's body mass corrected for body size, as recommended by Peig and Green (2009):

$$\text{Scaled mass index} = M_i(L_o/L_i)b_{\text{SMA}}$$

where M_i is the body mass of an individual i; L_i is the linear body measurement of an individual i (head-bill length) and b_{SMA} is the scaling exponent estimated from the regression of M and L. L_o is the arithmetic mean value of the linear measurement. We used the mean value of head-bill length for the target population. We chose the head-bill length for the linear body size measurement as this measurement was significantly correlated with the body mass in immatures (both sexes combined; Pearson's $r_{151} = 0.21, p = 0.009$) and adults (both sexes combined; $r_{65} = 0.47, p = 0.001$).

To describe the extent of moult observed for captured adults, we calculated a total moult score (TMS) for each individual. We determined TMS by adding the moult scores of individual primaries of the right wing, excluding the outermost primary because of its very small size (Ginn & Melville 1983), to give a maximum value of 45. This maximal score indicates complete replacement of all primaries. The minimum value (0) occurs when all feathers are old (unmoulted). For individuals with different scores in each wing (5 individuals), we calculated the mean moult score and used that in the analyses. In unmoulted adults, the TMS value was 0. Both sexes start and undergo the post-breeding moulting process within a similar time frame and rates of change (Kulaszewicz & Jakubas 2015).

To investigate factors affecting the H/L ratio, body condition (namely scaled mass index) and numbers of white blood cells (heterophils, lymphocytes and all leucocytes) per 10 000 RBC, we performed analysis of variance (ANOVA), with the mentioned variables as response variables (separate models for particular variables) and sex, age and trapping pentad as explanatory categorical factors. We also included interaction terms (sex × age, sex × trapping pentad, age × trapping pentad) in the model. Due to the absence or low numbers of captured birds during some days, we used grouped five-day periods (pentads; their definition and numbers after Busse 2000) instead of dates in the analyses. Before the analyses, we arcsin-square-root transformed the leucocyte variables and the H/L ratio (Zar 1999). Due to very low numbers or absence of monocytes, basophils and eosinophils in most of the blood smears, we excluded them from the analyses. As the variables did not differ between the studied years (Student's t-tests: all $p > 0.11$), we combined data from both years in all analyses. To investigate whether the time of day affects leukocyte profiles or body condition, we compared mentioned variables among three daily time periods of capture and sampling (hours 05:00–10:00; 10:00–15:00; 15:00–21:00).

There were no significant differences in leukocyte profiles or body condition among the intervals (ANOVA: all $p > 0.134$). Thus, we combined data from all hours, and neglected that factor in the analyses.

To investigate whether fat score is affected by (1) sex and age groups (explanatory variables), and (2) age and trapping pentad groups (explanatory categorical variables, and their interactions), we applied two separate distance-based permutational multivariable analyses of variance (PERMANOVA) based on the Bray-Curtis measure (Anderson 2001). We performed two separate analyses due to software constraints that limited analysis to two explanatory variables.

In adult birds, to investigate the influence of moult state [0 – individuals that did not changed any primary feather, 1 – individuals that changed at least one primary feather (80% of 66 adult individuals – 30 females and 23 males)] and sex (explanatory categorical variables) on leucocyte profiles (the H/L ratio or number of leucocytes per 10 000 RBC) and the scaled mass index, we performed ANCOVA with trapping pentad as a continuous variable, i.e. covariant (separate models for particular variables). We did not treat trapping pentad as an explanatory categorical variable because of the absence of non-moulting individuals (code 0) in a few trapping pentads.

Since main effects should not be interpreted when interactions are significant (*see*, for example, McDonald 2008), in such cases we interpreted only the interaction.

To examine relationships between leukocyte profiles or body condition and sex, age and pentad date, we build successive OLS linear regression models starting with multiple models. To find the best regression function, we did not use the stepwise method because several authors strongly recommend avoiding this procedure (reviewed in Dechaume-Moncharmont *et al.* 2011). Instead, we selected the best models using hierarchical regression based on the adjusted determination coefficient r^2 (Stanisz 2006). Only in the case of the H/L ratio, we initially conducted separate OLS regression analyses for immatures and adults since ANOVA revealed a significant influence of age. Models including only adults contained the H/L ratio or number of heterophils and lymphocytes as response variables and scaled mass index, sex, and TMS as explanatory variables.

To investigate the relationship between fat scores and (1) TMS and (2) trapping pentad in adult birds, we used Spearman's rank correlation. To investigate whether fat scores are affected by sex and moulting status and their interactions, we applied distance-based permutational multivariable analyses of variance (PERMANOVA) based on the Bray-Curtis measure (Anderson 2001). To compare fat scores between the two first and two last pentads, we used a Mann-Whitney *U*-test.

We performed PERMANOVA in PAST 2.17 (Hammer *et al.* 2001) and all other statistical analyses in STATISTICA 10.0 (StatSoft, Inc., Tulsa, OK, USA). The significance level was $p < 0.05$.

Results

Leucocyte profiles, scaled mass index and fat scores in relation to sex, age and trapping pentad

The H/L ratio was significantly affected by age, trapping pentad and the age × trapping pentad interaction. Neither sex, nor both the sex × age and sex × trapping pentad interaction affected the H/L ratio significantly (ANOVA, Table 1). Considering the age × trapping pentad interaction, adults had higher H/L ratios than immatures in pentads 42 and 43; the opposite pattern was recorded in pentads 46 and 47 (*post-hoc* HSD test for unequal *n*: $p < 0.03$). H/L ratios in adults and immatures were similar in the middle of the trapping pentad (pentads 44–45; 4–13 August; *post-hoc* HSD test for unequal *n*: $p > 0.05$). Finally, at the end of the trapping pentad, the H/L ratio in adults was significantly lower than in immatures (46–47; 14–23 August; *post-hoc* HSD test for unequal *n*: $p < 0.02$; Fig. 1). Among adults, the H/L ratio was significantly higher in pentads 42 and 43 compared to 46 and 47 (*post-hoc* HSD test for unequal *n*: $p < 0.02$). Immatures had similar H/L ratio values throughout the whole trapping pentad (*post-hoc* HSD test for unequal *n*: all $p > 0.12$; Fig. 1).

Relative numbers of heterophils, lymphocytes and leucocytes per 10 000 RBC were

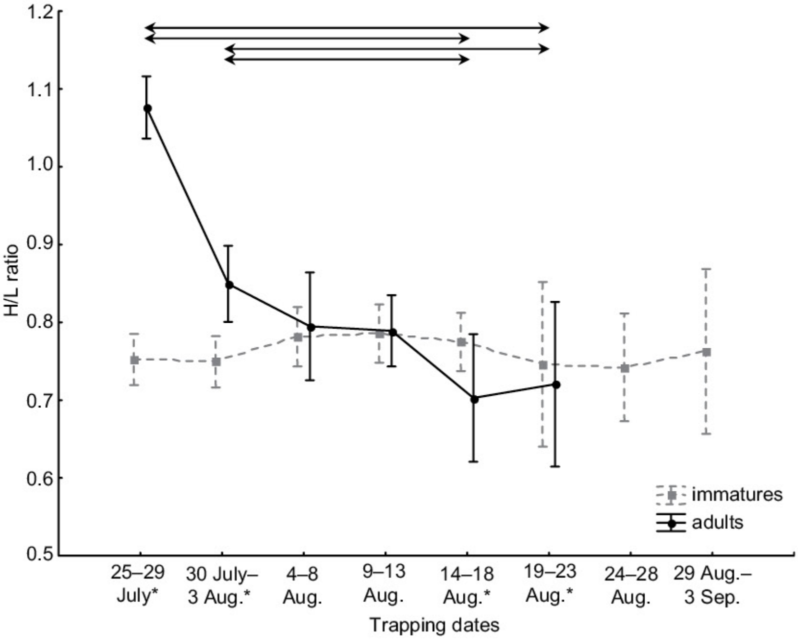

Fig. 1. The H/L ratio (mean ± SD) in adult and immature Savi's warblers captured during the post-breeding period. Asterisks indicate dates when significant age differences were found (ANOVA, *post-hoc* HSD Tukey test on transformed data, $p < 0.05$). Arrows indicate significant differences between pentads in adults (ANOVA, *post-hoc* HSD test for unequal *n* on transformed data, $p < 0.02$).

affected significantly by age, sex and their interaction (Table 2). Neither trapping pentad nor its interaction with sex and age influenced numbers of heterophils, lymphocytes and leucocytes per 10 000 RBC significantly. Regarding the age × sex interaction, adult females had higher relative number of leucocytes and heterophils and lower number of lymphocytes per 10 000 RBC than adult males. Immature females had lower numbers of leucocytes and heterophils than immature males. Immature females had higher relative numbers of leucocytes and heterophils per 10 000 RBC than adult males and females. Immature males and females had lower relative numbers of lymphocytes per 10 000 RBC than adult males (ANOVA, *post-hoc* HSD test for unequal *n*: $p < 0.03$; Table 2 and Fig. 2).

The scaled mass index was significantly affected only by the trapping pentad (Table 1). Individuals captured in pentads 42 and 43 (25–29 July and 30 July–3 August) had a higher scaled mass index than those captured in the last two pentads 48 and 49 (24–28 August and 29 August–2 September) (*post-hoc* HSD test for

Table 1. The effects of sex, age, trapping pentad and their interactions on the H/L ratio in Savi's warblers captured during the post-breeding period (factorial ANOVA on arcsin square-root transformed data); *p* values indicating significant effects are set in boldface.

Variable	H/L ratio			Scaled mass index		
	df	*F*	*p*	df	*F*	*p*
Intercept	1	11.43	< **0.001**	1	45.98	< **0.001**
Pentad	1	3.25	**0.035**	1	23.83	< **0.001**
Age	1	6.45	**0.011**	1	1.433	0.233
Sex	1	0.12	0.732	1	0.125	0.733
Pentad × age	1	6.03	**0.024**	1	3.98	0.132
Pentad × sex	1	1.85	0.112	1	2.243	0.168
Age × sex	1	0.38	0.546	1	0.924	0.171
Pentad × age × sex	1	1.63	0.154	1	1.276	0.214
Error	209			209		

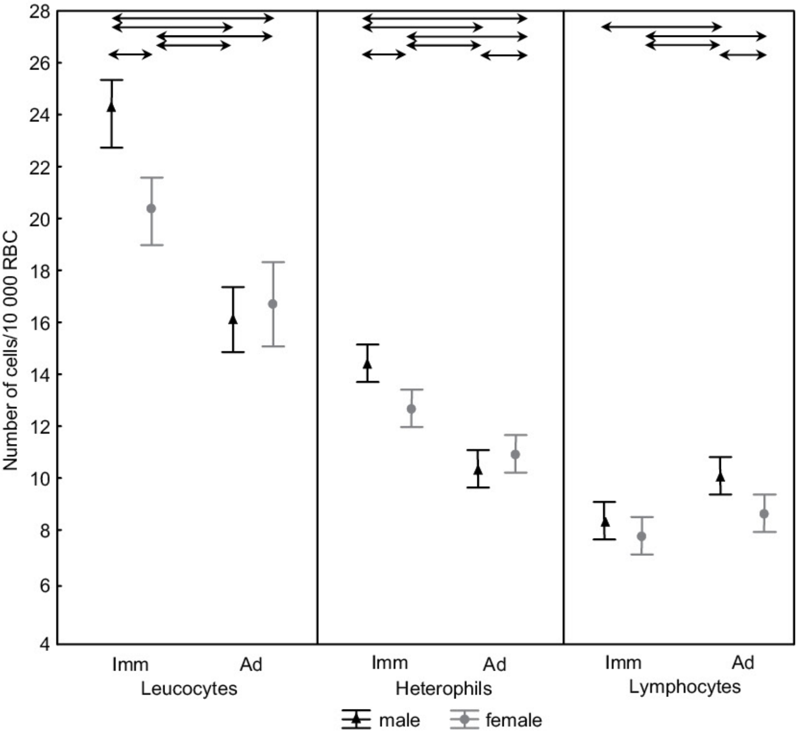

Fig. 2. Numbers of white blood cells per 10 000 RBC (mean ± SD) in males and females of adult and immature Savi's warblers captured during the post-breeding period. Arrows indicate significant sex × age interactions (ANOVA, *post-hoc* HSD test for unequal *n* on transformed data, $p < 0.05$).

unequal n: $p < 0.05$; Fig. 3). The scaled mass index was not affected significantly by the other factors, i.e. sex, age or the age × sex interaction.

PERMANOVA with fat score as response variable and age, sex and the age × sex interaction as explanatory variable revealed that only age affected fat scores significantly ($F_{1,207} = 12.94$, $p < 0.001$). Adults had higher fat scores (Median: 1, Q_1–Q_3: 0–1) than immatures (Median: 0, Q_1–Q_3: 0–1). Fat scores were not affected significantly by sex ($F_{1,207} = 0.18$, $p = 0.665$), and the age × sex interaction (PERMANOVA: $F_{1,207} = 0.487$, $p = 0.485$).

PERMANOVA with age, trapping pentad and their interaction as explanatory variables revealed that fat scores were affected significantly by age ($F_{1,213} = 15.13$, $p < 0.001$). Trapping pentad ($F_{1,213} = 3.65$, $p = 0.087$), and

Table 2. The effects of sex, age, trapping pentad and their interaction on numbers of heterophils, lymphocytes and leucocytes (all cells combined) per 10 000 red blood cells (RBC) in Savi's warblers captured during the post-breeding period (factorial ANOVA); *p* values indicating significant effects are set in boldface.

Variable	Leucocytes			Heterophils			Lymphocytes		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Intercept	1	22.94	< **0.001**	1	36.11	< **0.001**	1	67.98	< **0.001**
Pentad	1	3.87	0.065	1	4.89	0.057	1	3.21	0.059
Sex	1	12.62	**0.022**	1	6.79	**0.011**	1	4.95	**0.038**
Age	1	14.26	**0.003**	1	13.25	**0.001**	1	12.34	**0.002**
Sex × Age	1	10.77	**0.031**	1	7.29	**0.017**	1	6.52	**0.011**
Pentad × age	1	2.87	0.074	1	3.65	0.063	1	2.24	0.072
Pentad × sex	1	2.02	0.072	1	2.12	0.097	1	2.56	0.069
Pentad × age × sex	1	3.11	0.084	1	4.04	0.061	1	2.14	0.092
Error	209			209			209		

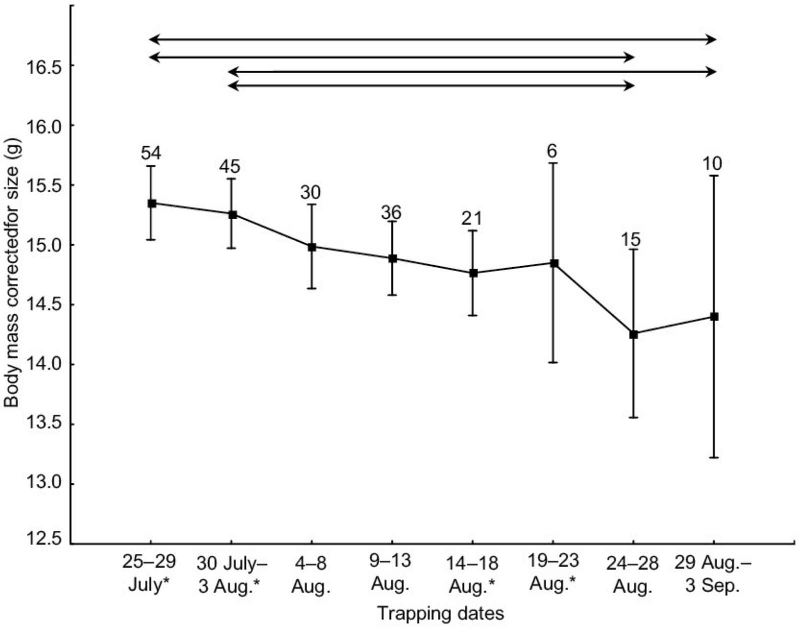

Fig. 3. Body mass corrected for size using the scaled mass index (mean ± SD) in Savi's warblers captured during the post-breeding period. All age and sex categories combined. Arrows indicate significant differences between dates (ANOVA, *post-hoc* HSD test for unequal *n*, $p < 0.05$), numbers above indicate sample sizes.

the trapping pentad × age interaction ($F_{1,213} = 4.12$, $p = 0.072$) did not affect the fat reserves observed in our study significantly.

Due to differences in the scaled mass index among trapping pentads, we compared fat scores between individuals captured during pentads 42–43 combined (25 July–3 August) and 48–49 combined (24 August–2 September). Birds captured during the two first pentads had significantly lower fat scores (Median: 1, Q_1–Q_3: 1–2) as compared with those caught during the two last pentads (Median: 0, Q_1–Q_3: 0–1; Mann-Whitney *U*-test: $Z = 5.16, p < 0.005, n_{\text{period 1}} = 80$, $n_{\text{period 2}} = 25$).

Effect of moult on leucocyte profiles and the scaled mass index in adults

ANCOVA revealed that in adult Savi's warblers, the H/L ratio was affected significantly by the covariant trapping pentad (ANCOVA: $F_{1,61} = 45.01, p < 0.01$) and moult state (ANCOVA: $F_{1,61} = 33.80$, $p < 0.01$). Moulting individuals had lower H/L ratios (mean ± SD: 0.83 ± 0.015, $n = 53$) than non-moulting individuals (mean ± SD: 1.07 ± 0.035, $n = 13$). Neither sex nor the moult × sex interaction affected the H/L ratio significantly (Table 3).

Moult and trapping pentad also influenced the number of leucocytes per 10 000 RBC significantly (ANCOVA, Table 3). Trapping pentad influenced the number of leucocytes (ANCOVA, $F_{1,61} = 10.34; p < 0.001$), heterophils (ANCOVA: $F_{1,61} = 12.54$, $p < 0.005$) and lymphocytes (ANCOVA: $F_{1,61} = 45.01, p < 0.001$). Moulting individuals had higher numbers of leucocytes, heterophils and lymphocytes (mean ± SD: leucocytes: 18.2 ± 1.36; heterophils: 12.3 ± 0.96; lymphocytes: 11.8 ± 1.05) than non-moulting individuals (mean ± SD: leucocytes: 14.4 ± 3.67; heterophils: 10.2 ± 2.81; lymphocytes: 9.2 ± 2.86). Neither sex nor the moult × sex interaction affected the relative number of leucocytes, heterophils and lymphocytes per 10 000 RBC significantly.

The scaled mass index was affected significantly by trapping pentad (ANCOVA: $F_{1,61} = 6.85$, $p = 0.034$). Neither moult nor sex or the moult × sex interaction had a significant effect on the scaled mass index (Table 3).

PERMANOVA with sex and moult status as explanatory variables revealed that fat scores in adults were not significantly affected by sex ($F_{1,61} = 3.11$, $p = 0.11$), moulting status ($F_{1,61} = 5.67$, $p = 0.24$), or by the sex × moulting status interaction ($F_{1,61} = 4.82, p = 0.44$).

Relationships between the studied variables

In adults, the best function revealed that the H/L ratio decreased with increasing TMS values expressing the progress of moult (Pearson's $r_{65} = -0.71$, adjusted $r^2 = 0.58$, $p < 0.001$; Fig. 4). Trapping pentad was excluded from the analysis due to its correlation with TMS; Pearson's $r_{65} = 0.43$, $p = 0.004$). In immatures, the best regression model was insignificant ($p = 0.28$, $r^2 = 0.11$). The H/L ratio was negatively correlated with trapping pentad (Pearson's $r_{65} = -0.68$, $p < 0.001$).

In adults, hierarchical regression revealed two functions that describe the best relationship between the number of leucocytes per 10 000 RBC and variables included in the model (scaled mass index, sex, TMS). The advance of moult (higher TMS) decreased with an increase in the number of heterophils (Pearson's $r_{65} = -0.68$, adjusted $r^2 = 0.48$, $p < 0.001$) and an increase in the number of lymphocytes ($r_{65} = 0.49$, adjusted $r^2 = 0.42$, $p < 0.001$). Trapping pentad was excluded from the analyses because it was redundant (correlation with TMS; Pearson's $r_{65} = 0.51$, $p = 0.008$). Trapping pentad was negatively correlated with the number of leucocytes (Pearson's $r_{65} = -0.60$, $p < 0.001$), heterophils (Pearson's $r_{65} = -0.48$, $p < 0.001$) and lymphocytes per 10 000 RBC (Pearson's $r_{65} = -0.37$, $p = 0.002$). There were no other significant relationship between numbers of heterophils and lymphocytes and sex or the scaled mass index (the best regression function $p = 0.23$).

Hierarchical regression revealed one function describing the best relationship between the scaled mass index and studied variables in all individuals. This function revealed that scaled mass index decreased significantly with pentad (Pearson's $r_{199} = -0.32$, $r^2 = 0.41$, $p < 0.001$).

In adults, there was no significant relationship between fat scores and TMS (Spearman's $r_s = 0.21$, $p = 0.103$, $n = 66$) nor between fat scores and trapping pentad (Spearman's $r_s = 0.154$, $p = 0.187$, $n = 66$).

Discussion

To our knowledge, this is the first study investigating leucocyte profiles in the Savi's war-

Table 3. The effects of moulting and sex, and the sex × moulting interaction on numbers of leucocytes, heterophils, lymphocytes per 10 000 red blood cells, the H/L ratio and the scaled mass index in adult Savi's warblers captured during the post-breeding period (ANCOVA with trapping pentad as covariant); *p* values indicating significant effects are set in boldface.

Variable	Leucocytes			Heterophils			Lymphocytes		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Intercept	1	29.76	< **0.001**	1	6.64	**0.012**	1	12.56	< **0.001**
Pentad	1	10.34	**0.002**	1	12.54	**0.005**	1	5.84	**0.018**
Moulting	1	33.28	< **0.001**	1	34.60	< **0.001**	1	7.38	**0.008**
Sex	1	8.35	**0.006**	1	5.43	**0.044**	1	8.32	**0.009**
Sex × moulting	1	1.45	0.234	1	0.31	0.570	1	4.19	0.076
Error	61			61			61		

Variable	H/L ratio			Scaled mass index		
	df	*F*	*p*	df	*F*	*p*
Intercept	1	35.22	< **0.001**	1	8.21	**0.012**
Pentad	1	45.01	< **0.001**	1	6.85	**0.034**
Moulting	1	33.80	< **0.001**	1	0.14	0.712
Sex	1	2.26	**0.008**	1	0.02	0.913
Sex × moulting	1	1.68	0.214	1	0.22	0.644
Error	61			61		

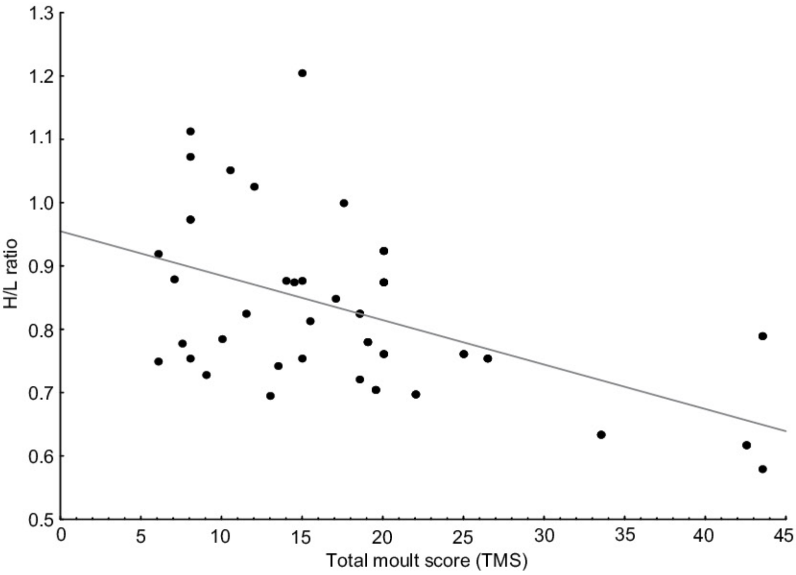

Fig. 4. The relationship between values of the H/L ratio and total moult score of primaries (TMS) in moulting adult Savi's warblers (Pearson's correlation, $r_{65} = -0.71$, $p < 0.001$).

bler. We found that adults had a higher H/L ratio than immatures during the first two trapping pentads (42–43; 25 July–3 August). This decrease in the H/L ratio with date suggests that elevated values in adults at the beginning of our study may represent a carry-over effect from the breeding season, when the reallocation of energy and nutrients from immune function to reproduction is often reported (Lochmiller & Deerenberg 2000). Effects of these physiological adjustments may be visible in leucocyte profiles even after the breeding season (Norris & Evans 2000). A gradual decrease of the H/L ratio in adults probably reflects post-breeding relaxation of physiological stress after reproduction, or progress of moulting which usually affects leukocyte profiles (*see* below).

Adult females had higher numbers of leucocytes and heterophils per 10 000 RBC and lower number of lymphocytes compared to adult males (Table 2 and Fig. 2). Males and females in the Savi's warbler are assumed to share parental duties equally (Pikulski 1986). However, as the breeding biology of the Savi's warbler has not been studied in great detail (Aebischer *et al.* 1996, Neto & Gosler 2005), it is possible that there are subtle sex differences in parental care at the end of breeding period.

Most of the adult Savi's warblers caught in the present study had started their post-breeding moult. During the first two trapping pentads of the study, only 36% of adults that we caught had not started to moult (Kulaszewicz & Jakubas 2015). Moulting adults had significantly lower H/L ratios as compared with non-moulting birds. Moreover, the H/L ratio decreased with the advancement of moult. Lower values of the H/L ratio in moulting individuals have also been reported for other passerines (e.g. Driver 1981, Hawkey *et al.* 1985, Sanz *et al.* 2000, Jakubas *et al.* 2011). The lower values of the H/L ratio together with elevated numbers of leucocytes, heterophils and lymphocytes per 10 000 RBC suggest efficient immune function (De Casto & Timmis 2002). The "standby mode" of the immune system is activated to avoid various forms of infection (e.g. bacterial) of wounds resulting from breakage of growing new feather follicles (Silverin *et al.* 1999, Fokidins *et al.* 2008). Decreasing values of the H/L ratio with the progress of moult seems to indicate the absence of a trade-off between moult and the immune system. Moult can be energetically expensive for an organism. Evidence of this has been shown in experimental studies on the house sparrow *Passer domesticus* and chicken *Gallus gallus domesticus*, revealing a trade-off between moulting and the immune system, when after induced moulting — the H/L ratio increased throughout the moulting process (Holt 1992, Alodan & Mashaly 1999, Davis *et al.* 2000, Martin 2005, Moreno-Rueda 2010). In our study, conducted in natural conditions, such a trade-off was not observed. Birds with sufficient resources may mount an immune response at the same time as they moult efficiently (Borras *et al.* 2004, de la Hera *et al.* 2009). Birds without sufficient resources would have a limited capacity to combat pathogens, increasing mortality (Møller & Saino 2004), and/or they would moult plumage during bad conditions, which would affect their future fitness (Nilsson & Svensson 1996). The moulting process in the Savi's warbler is extended in time, but also often suspended (Neto & Gosler 2006, Kulaszewicz & Jakubas 2015). A suspended strategy of moult may be less energy demanding per time unit, resulting in a lack of the trade-off between moult and the immune system.

The observed decrease of the scaled mass index with capture date may be explained by inter-seasonal changes in the proportion of captured birds representing local and migratory individuals. The birds caught late in the season with lower body mass and far scores are less likely to be the local breeding birds, and more likely represent transient northern populations using our study area as a stopover site to refuel before further migration. Alternatively, a decrease in the scaled mass index with trapping pentad and differences in fat scores between first and last pentads may be an effect of declining food resources at the stopover site. Local concentrations of migrating, fat-depleted birds sharing dietary preferences can lead to increased competition for food at the time of elevated energy demands (Moore & Yong 1991). Savi's warblers may compete for food with reed warblers (*Acrocephalus scirpaceus*) and sedge warblers (*A. schoenobaenus*), which occur in high numbers in the study area during their autumn migration (Jakubas & Wojczulanis-Jakubas 2010).

Higher fat reserves in adults as compared with those in immatures might be explained in the context of more efficient foraging of experienced and/or higher social status adults (Woodrey & Moore 1997). The discrepancy between results for the scaled mass index and fat scores might be accounted for by the low resolution of the fat score system used and/or a nonlinear relationship between fat mass and fat score (Hedenström *et al.* 2009). Also, not all energy reserves used during migration are accumulated in fat, but may also be as proteins (Lindström & Piersma 1993).

In conclusion, we found that the age × pentad and age × sex interactions significantly affected the H/L ratio or numbers of various leukocytes per 10 000 RBC. The process of moulting also significantly affected leukocyte profiles and body energy reserves in Savi's warblers. However, in contrast to reports from other species studied, we did not find evidence for the existence of a trade-off between immune function and moulting in the Savi's warbler.

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